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Applicant: FODSTAD, Øystein et al	

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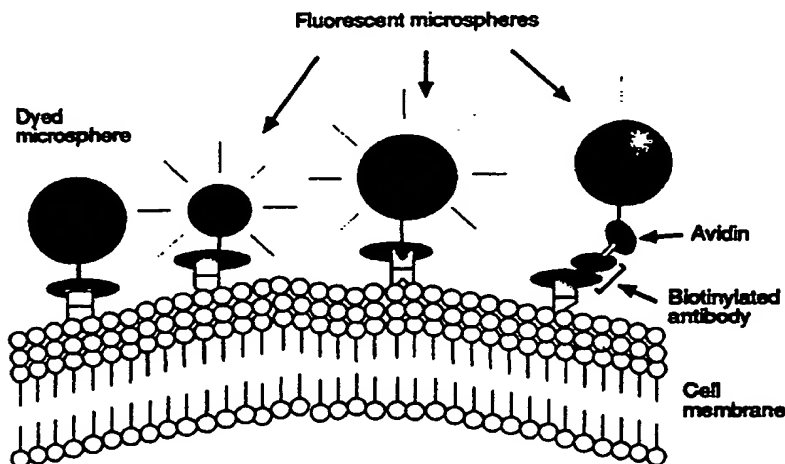
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(54) Title: METHOD FOR CHARACTERIZATION OF ABNORMAL CELLS



(57) Abstract

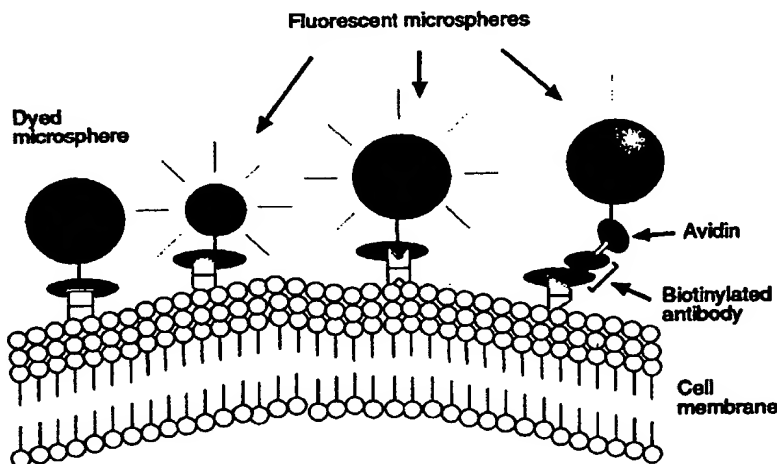
It is described a method to detect and phenotype target cells in cell suspensions by using particles coated with antibodies/ligands directed to antigenic determinants/receptors expressed on the target cells, characterized in that several types of particles, each particle instrumentally or visually separable by fluorescence, color and size, and each particle coated with a different antibody or ligand, are incubated simultaneously or subsequently with cell suspensions containing the target cells, in connection or not with a previously known enrichment procedure, a use of the method and a kit.



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(54) Title: METHOD FOR CHARACTERIZATION OF ABNORMAL CELLS



## (57) Abstract

It is described a method to detect and phenotype target cells in cell suspensions by using particles coated with antibodies/ligands directed to antigenic determinants/receptors expressed on the target cells, characterized in that several types of particles, each particle instrumentally or visually separable by fluorescence, color and size, and each particle coated with a different antibody or ligand, are incubated simultaneously or subsequently with cell suspensions containing the target cells, in connection or not with a per se known enrichment procedure, a use of the method and a kit.

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## Method for characterization of abnormal cells

The present invention relates to a new method for identification and characterization of eukaryotic cells.

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In several diseases such as primary and secondary malignancies, allergic, autoimmune, inflammatory, proliferative, infectious, and destructive disorders, or diseases for which the underlying mechanisms are unclear, it would be of utmost importance to be able to determine as many as possible characteristics of cells involved in the pathologic processes. An exact determination of a number of different markers on such cells would significantly improve diagnosis, prognostication and the choice of subsequent therapy. If such a procedure is simple and rapid, it would be easy to diagnose pathological conditions at an early stage of the disease, thereby increasing the probability of selecting the best therapeutic alternative at a time when the treatment may be most effective. Moreover, in some situations it is of crucial importance to make an immediate and correct diagnosis, such as to distinguish between a benign and a malignant tumor, to guide in the surgeons' selection of a proper operative procedure.

Presently, the following diagnostic methods related to the above mentioned pathological conditions are at hand: Conventional morphological examination of tissue sections, cell cytopins or smears, and immunological methods including immunocytochemistry, flowcytometry, and immunofluorescence microscopy. In addition, peroperative morphological evaluation of biopsied tissue specimens are performed on frozen sections.

With the non-immunological methods, the diagnosis can only provide distinction between normal and pathological cells based on morphological criteria.

Immunological methods such as immunocytochemistry and flowcytometry represent valuable diagnostic tools, although they suffer from several important limitations. With both methods, heterogenous cell populations are exposed to antibodies or other ligands for their binding to target cells. For flowcytometry studies live or fixed cells may be incubated to allow for

fluorescence-labeled antibodies to bind to relevant membrane or intracellular antigens, before the cell suspension is analyzed in the instrument. Immunocytochemistry requires preparation of tissue sections, cytopins or smears, fixation and immunostaining of the cells before evaluation in a microscope. Visualization of bound antibodies is obtained indirectly through one or several steps ending with an enzyme/substrate color reaction, allowing the stained cells to be observed in a microscope. The multi-step procedure can not be completed on the day of cell sampling. Moreover, usually thorough evaluation by an experienced pathologist is needed for obtaining reliable results. For example, if the abnormal cells are being identified in a mixed cell population, and the ratio of pathological to normal cells is low, such as malignant cells in samples of bone marrow or peripheral blood, an excessive amount of work performed by a skilled pathologist may be needed for cell identification. Another problem is related to the fact that there are very few antibodies recognizing antigens that are selectively and consistently expressed in all target cells. If the objective is to identify tumor cells in blood and bone marrow, antibodies directed against "tissue-specific" markers, such as cytokeratins found in epithelial cells, are commonly used. However, as it is known that some normal cells may also express cytokeratins and that not all malignant epithelial cells do, there is a risk of both false positive and negative results.

Fluorescence-labeled antibodies can be used to detect target cells either by fluorescence microscopy or by flow cytometry. The former procedure can successfully be employed to demonstrate binding of a single antibody, although the use of morphological criteria as an additional way of distinguishing between normal and pathological cells is very limited. Moreover, the fluorescence usually fades and disappears rapidly under examination in the microscope. Thus, it is required that the fluorescence cells are studied and assessed microscopically within a short timeframe after binding of the antibody. Flowcytometric analysis requires the presence of a high number of target cells to provide reliable results. Moreover, the procedure does not provide any possibilities for morphological studies or for distinguishing between fluorescent target and non-target cells. Furthermore, several mentioned methods have the disadvantage that cells are lost in the methodological steps.

Improved possibilities for detecting target cells have recently been described (WO94/07138, WO94/07139, WO95/24648). In these procedures, antibodies bound to super-paramagnetic particles are used for detection and selection of the cells to be identified. One limitation of these methods is that it can be difficult to prove directly the pathological nature of cells with bound particles on the surface. One advantage compared to the other described methods is, however, the simplicity of the procedure and that results can be obtained within a very short timeframe.

To further confirm the pathologic nature of stained, fluorescent or immunobead-binding cells it is highly warranted to characterize the target cells for more than one marker. The aim is to obtain important biological information, and information of crucial diagnostic and prognostic significance. If the number of target cells is high, flow cytometry may be used to study in parallel the binding of two different cell-bound antibodies. However, this method lacks the possibility to examine individual cells and cell morphology, and actually to identify fluorescent cells as the real target cells. Immunocytochemistry does allow for a maximum of two markers to be studied in parallel, one with conventional enzymatic visualization of bound antibody and one with a silver/gold enhancement procedure. Both multi-step procedures are relatively complicated, time-consuming and requires either expensive equipment and/or special expertise in the respective areas.

With the immunomagnetic method, further characterization of whole cells may be obtained by preparing cytopspins of the magnetically selected cells and thereafter performing immunostaining as for conventional immunocytochemistry. Therefore, the same limitations as described for immunocytochemistry apply, and furthermore because the target cells have beads attached to their surface it may be difficult to get the cells to stick to the slides used for conventional cytopspin preparation.

In conclusion, the existing methods provide possibilities for studying a maximum of two independent markers, and inherent to the described methods several important problems and limitations are present. It was therefore highly warranted to develop a method that much more simply, rapidly, and

reliably could help identifying and characterizing target pathological cells. The complexity and heterogeneity of cell biology makes it also warranted to be able to examine expression of several independent biological markers on the same cells. Such biological information would be of vital diagnostic and prognostic significance that can aid in the choice of therapeutic alternative. When several markers are examined in parallel it would be possible to obtain a more reliable confirmation of the pathological nature of the target cells, thereby improving the diagnostic reliability and help excluding false positives as well as negatives. Importantly, multi-parameter characterization could include markers of cell proliferation, cell death (such as apoptosis), adhesion, motility, invasion, antigenicity, inflammation, cell destruction, auto-immune mechanisms, angiogenesis, disease aggressiveness, tumor metastasis, and inhibitors of all these functions. Furthermore, if several markers can be examined also at an individual cell level, it would be possible to study cell heterogeneity and identifying subsets of cells with specific biological properties. In some diseases it would also be important to study pathological cells obtained from different sites in the same patient in order to determine whether cell characteristics could vary from one site to another, thus providing additional biologically and prognostically important information. Altogether, the impact of obtaining information of the type here described for clinical evaluation and treatment of patients can hardly be overestimated.

These objects are obtained in the present invention as characterized by the enclosed claims.

We here introduce a new concept in characterization of intact target cells in cell suspensions, making a direct microscopical identification of more than two membrane-associated markers possible. With this method several cell membrane markers of the origin, biology and potential fate of target cells can be studied in the same operation. The procedure is very simple and can be completed within a very short timeframe without the need for advanced and expensive instrumentation. With the method, target cells can with a minimum of handling steps, without any cell loss, be studied microscopically for the expression of several independent marker molecules, even at the individual cell level. Thus, in addition to obtaining an overall picture of biological



parameters present in the target cell population, such a procedure also allows for examining cell to cell variation in the expression of marker molecules, providing information with vital biological and medical implications.

5 Briefly, the method can be performed as here described: Dyed or fluorescent microspheres (beads, particles) conjugated with antibodies or ligands that can bind to cell membrane determinants to be studied are added to the cell suspension and incubated under gentle rotation. Thereafter, samples of the cell suspension are examined in a fluorescence microscope for cells with  
10 surface-bound microspheres of different light or fluorescent colors. The extent and variation in cell binding of the different microspheres can be assessed and quantitated. The assessment of cell-bound particles may, if warranted, be performed by an automated procedure.

15 In the following the present invention is described in greater detail with the examples, which by no means are intended to restrict the invention, and figures in which:

Fig. 1 illustrates the binding of four types of microspheres to four different  
20 antigenic determinants expressed on the membrane of a target cell. The binding is in this case mediated through four different antibodies, each recognizing one of the said four antigens, in that the antibodies had first been conjugated to the respective microspheres, either directly through a chemical bond (Fig. 1, the three examples to the left), or indirectly where the beads  
25 had been pre-coated with avidin before conjugation to a biotinylated antibody (Fig. 1, right example). As illustrated in Fig.1, one antibody was bound to a blue dyed microsphere, one antibody to a small and another to a larger red fluorescent microsphere, whereas the fourth antibody had been biotin-avidin conjugated to a green fluorescent microsphere.

30 Fig. 2 illustrates how the invention can be used to characterize two or more cell membrane determinants in a situation where the target cells in a mixed cell suspension are rare, thus warranting an enrichment procedure before evaluation of the sample. In the illustrated case, antibody coated  
35 superparamagnetic beads are bound to the cell membrane together with red and green fluorescent microspheres conjugated either directly or through an

avidin-biotin binding to separate antibodies. In such a situation, immunomagnetic enrichment can be obtained by using a strong magnet that will attract cells with bound magnetic beads. The enriched cell suspension is thereafter examined in a microscope in which the binding of the fluorescent microspheres to target cells with bound magnetic beads can be observed.

The visually or instrumentally different dyed or fluorescent particles, which can be of similar or different sizes, used in the invention are conjugated to ligands such as antibodies, or fragment of antibodies, lectins and growth factors, that can bind to specific molecules expressed on membranes of abnormal cells, so that the bound particles can be identified microscopically. Examples include the use of polystyrene latex fluorescent microspheres of various colors that can be observed in a fluorescence microscope, and dyed non-fluorescent particles, such as red, yellow, green, black and blue, that can be detected by light microscopy. Antibodies conjugated to the microspheres include all those recognizing antigens, receptors, and other determinants expressed on membranes of abnormal cells, and on normal cells, see below. By combining different antibody-particle conjugates relevant for the cells to be studied, a finger-print of cell characteristics can be obtained rapidly and directly in the cell suspension. Such antigenic finger-prints would be highly valuable in evaluating important biological characteristics of cells, see above, cell populations or sub-populations. The simplicity and speed by which the method can provide such information is surprising and constitutes a key element of the invention.

Antibody-conjugated fluorescent and dyed particles have been used in various types of immunoassays to determine, e.g. the presence of free antigens, proteins, viruses and bacteria in biological fluids. With intact eukaryotic cells, fluorescent microspheres conjugated to antibodies have been used to study in each case a single molecule expressed in a specific type of normal cells, such as monocytes, lymphocytes, hepatocytes and fibroblasts. The purpose of these studies have been such as examination of the motility of membrane markers in macrophages or metabolic parameters in hepatocytes. There is no report found in the literature on attempts to study abnormal cells, such as malignant and benign neoplastic cells, and abnormal cells found in various infectious, reactive, autoimmune, inflammatory and

proliferative disorders. Furthermore, combination of several antibodies conjugated to different dyed or fluorescent microspheres on the same cell population, or on individual cells, are not described. Also, such procedures have not been employed to study, for biological or diagnostic purposes, sub-

5 populations of target cells in a mixed population of cells.

The particles to be used can be fluorescent polystyrene latex microspheres or non-fluorescent particles of different colors. The size of the microspheres can be between 0.01  $\mu\text{m}$  and 6  $\mu\text{m}$ . The particles should provide possibilities for

10 conjugating antibodies or other ligands to their surface. This may be obtained directly, such as through chemical groups like carboxyl, amino or other groups, or indirectly by binding antibodies to microspheres previously coated with proteins such as avidin, streptavidin, protein A, or with antibodies that can react with a second antibody. The size of the microspheres may be

15 chosen to fit the size of the cells and the purpose of investigation, such that it would facilitate identification of different bound antibody-microsphere conjugates. It is considered that a particle size of e.g. 1  $\mu\text{m}$  makes identification of a relatively low number of bound particles easy, whereas a smaller size may possibly be advantageous if a marker protein expressed at

20 high density is to be studied. Another important feature of the invention is that it can be applied both when a very low or a very high number of cells are to be examined. It is also important that the fluorescent microspheres can retain their fluorescence strength for a considerable length of time.

The antibodies recognizing the relevant membrane marker antigens or receptors could either be whole IgG of any isotype, IgM antibodies or any fragments of such antibodies, including also recombinant antibodies or antibody fragments. The novel method includes binding of the said

25 fluorescent or dyed microspheres to target cells in a suspension with a low number of non-target cells, and in other cases where the number of target cells is low compared to non-target cells. The cell suspension is incubated with several antibodies, preferably 2-6, each conjugated to different microspheres, of the same or different sizes, of a specific dye or fluorescent

30 color. The ratio between the number of particles and the number of target cells ranges from 20:1 to 0.5:1, preferably 5:1, limited by the size of the

35 particles. The cell suspension should be incubated with antibody-coated

beads for 5-10 minutes to 2 hours, preferably for 30 minutes, at 0°-37°C, preferably at 4°C under gentle rotation. After incubation, samples of the cell suspension is taken for evaluation in a fluorescence microscope or in other visualizing or imaging devices in which fluorescent particles and dyed particles can be observed. Microspheres that are bound to cells can then be visualized, and the number of cells with the different types of particles attached to their surface can be assessed, with or without enumeration also of the number of beads attached to the cells. Since it is possible to use a combination of several antibody-coated microspheres, fluorescence filters suited to study different fluorescence emission spectra may be used. The method also provides possibilities for semi-automatic, video, and computer image analysis of the presence of dyed or fluorescent particles bound to the cells.

The antibodies could be of murine, rat, rabbit or human origin and may preferably recognize antigens present on target cells and not on normal cells in mixed cell suspensions. A list of antibodies/ligands includes, but are not limited to, those directed against groups of antigenic determinants, for example CD56/NCAM antigen, pan-epithelial EGP2/cluster2 antigen, breast mucin (MUC1) and other mucin epitopes, HMW and other melanoma-associated antigens such as gp100, MAGE 1,2 and 3, and MUC18, 80kD sarcoma associated antigen, erbB2, receptors for growth factor such as EGF, TGF, PDGF, bFCF, VEGF, IGF1, and IGF2, laminin, laminin5, uPA, uPAr, PAI, TIMP1 and 2, stromelysin, and other invasion related molecules, CEA, PSA, PSM, NSE, c-Met, CD44 and variants, ICAM-1, integrins, cadherins, catenins and other cell adhesion-associated molecules, drug resistance markers such as MDR and MRP, apoptosis-related molecules such as Fas and FasL, markers of cell proliferation, motility, differentiation, metastasis, angiogenesis, signal transduction, and inflammation-related membrane molecules, oncogene products, and chemokine receptors such as CCR 1-5, CXCR 1-4, and Duffy antigen, and all types of hematopoietic and lymphatic cell markers categorized in the CD system. Table 1 lists groups of membrane determinants that can be targeted and a number of examples within each group is also presented.

Table 1.

Antigens/receptors and corresponding antibodies/ligands

<b>ANTIGENS/RECEPTORS</b>	<b>EXAMPLES OF ANTIBODIES/LIGANDS</b>
<b>Adhesion molecules</b>	
Integrins	Pierce 36114, BTC 21/22, M-Kiol 2 BTC 41/42, Calbiochem 407277-84
ICAM-1 (CD54)	C57-60, CL 203.4
VCAM-1	Genzyme 2137-01
NCAM (CD56)	MOC 1
HCAM	BCA 9
LCAM	BM 1441 892
CD44	BM 1441 272, 25.32
CD44 variants	11.24, 11.31
ELAM-1	Genzyme 2138-01
E-selectin	BBA 8
P-selectin	BTC 71/72
LFA-3 (CD58)	TS 2/9
MACAM-1	NKI-M9
E-cadherin	BTC 111, 6F9
P-cadherin	NCC-CAD-299
Tenascin	BM 1452 193
Thrombospondin receptor (CD36)	BM 1441 264
VLA-2	A1.43
<b>Carbohydrate antigens</b>	
T-antigen	HH8, HT-8, Lectins
Tn-antigen	TKH6, BaGs2, Lectins
Sialyl Tn	TKH-2
Galbl-4GlcNac (nL4, 6, 8)	1B2, Lectins
Gastrointestinal cancer associated	

antigen (M.200kD)	CA 19-9
Le <sup>y</sup>	MLuC1, BR96, BR64
di-Le <sup>x</sup> , tri-Le <sup>x</sup>	B3
CA15-3 epitope	CA15-3
CEA	I-9, I-14, I-27, II-10, I-46,
Lacto-N-fucopentannose III (CD15)	PM-81

### **Glycolipids**

GD <sub>3</sub>	ME 36.1, R24
GD <sub>2</sub>	ME 36.1, 3F8
Gb <sub>3</sub>	38-13
GM <sub>3</sub>	M2590
GM <sub>2</sub>	MKI-8, MKI-16
FucGM <sub>1</sub>	1D7, F12

### **Growth factor receptors**

EGF receptor	425.3, 2.E9, 225
c-erbB-2 (HER2)	BM 1378 988, 800 E6
PDGF $\alpha$ receptor	Genzyme 1264-00
PDGF $\beta$ receptor	Sigma P 7679
Transferrin receptor	OKT 9, D65.30
NGF receptor	BM 1198 637
IL-2 receptor (CD25)	BM 1295 802, Bm 1361 937
c-kit	Bm 428 616, 14 A3, ID9.3D6
TNF-receptor	Genzyme 1995-01, PAL- M1
NGF receptor	

### **Melanoma antigens**

High molecular weight antigen (HMW 250.000)	9.2.27, NrML5, 225.28
Mw105 melanoma-associated glycoprotein	ME20
100 kDa antigen (melanoma/carcinoma)	376.96
gp 113	MUC 18

p95-100	PAL-M2
gp75/TRP-1	15.75, TA99
gp 100-107	NKI-beteb
MAA	K9.2
M125kD (gp125)	Mab 436
MAGE 1, 2, 3	anti-MAGE 1, 2, 3
Tyrosinase	anti-tyrosinase

**Sarcoma antigens**

TP-1 and TP-3 epitope	TP-1, TP-3
M.200kD	29-13, 29.2
M.160.kD	35-16, 30-40

**Carcinoma markers**

EGP-2 (cluster 2 epithelial antigen)	MOC-31, NrLu10
MUC-1 antigens (such as DF3-epitope (gp290kD)	BM7, DF3, BCP-7 to -10
MUC-2 and MUC-3	PMH1
LUBCRU-G7 epitope (gp 230kD)	LUBCRU-G7
Prostate specific antigen	BM 1276 972
Prostate cancer antigen	E4-SF
Prostate high molecular antigen M.> 400kD	PD41
Polymorphic epithelial mucins	BM-2, BM-7, 12-H-12
Prostate specific membrane antigen (Cyt-356)	7E11-C5
Human milk fat globulin	Immunotech HMFG-1, 27.1
42kD breast carcinoma epitope	B/9189
M <sub>w</sub> > 10 <sup>6</sup> mucin	TAG-72, CC-49, CC-83
Ovarian carcinoma OC125 epitope (m. 750 kD)	OC125, OVX1
Pancreatic HMW glycoprotein	DU-PAN-2
Colon antigen Co-17-1A (M.37000)	17-1A
Ga 733.2	GA733, KS1.4

TAG 72	B72.3, CC-49, CC-83
Pancreatic cancer-associated	MUSE 11
Pancarcinoma	CC49
Prostate adenocarcinoma-antigen	PD 41
M <sub>w</sub> 150-130kD adenocarcinoma of	AF-10
M <sub>w</sub> 92kD bladder carcinoma	3G2-C6
M <sub>w</sub> 600kD bladder carcinoma	C3
Bladder carcinoma antigen	AN43, BB369
Hepatocellular carcinoma antigen	KM-2
M.900kD	
M <sub>w</sub> 48kD colorectal carcinoma	D612
Colon specific antigen	Mu-1, Mu-2
Lung carcinoma antigen M. 350-420kD	DF-L1, DF-L2
Colon cancer-associated	C242, NCRC37
Bladder carcinoma antigens	T16, T43, T138

#### **Neuroblastoma antigen**

Neuroblastoma-associated, such as UJ13A

epitope	UJ13A
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#### **Glioma antigens**

Mel-14 epitope	Mel-14
HMW 250kD	9.2.27

#### **Head and neck cancer antigens**

M.18-22kD antigen	M48
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#### **HLA-antigens**

HLA Class 1	TP25.99
HLA-A	VF19LL67
HLA-B	H2-149.1
HLA-A2	KS1
HLA-ABC	W6.32
HLA-DR, DQ, DP	Q 5/13, B 8.11.2
β <sub>2</sub> -microglobulin	NAMB-1



**Apoptosis associated molecules**

Fas (CD95/APO-1)	Apo 1
FasL	Anti-FasL
P75	NGF

**Various**

cathepsin D	CIS-Diagnostici, Italy
neuroglandular antigen (CD63)	ME91, NKI-C3, LS62
pan-human cell antigen	pan-H

**Motility related antigens**

anti-KAI-1, anti-AMF

**Proliferation-associated markers**

anti-gp120, anti-Ki-67

**Differentiation-associated markers**

MUC 18, TA99

**Drug resistant-related markers**

C 219, MRK 16, anti-MRP

**Angiogenesis-associated markers**

anti-VEGF, anti-bFGF

**Chemokine receptors markers**

anti-CCR, anti-CXCR

**Invasion-related antigens**

Antibodies to:

PAI, MMP1, MMP9,  
TIMP1, TIMP2,  
laminin V, stromelysin,  
uPAR, uPA

**B- and T-cell CD antigens**

HH1, AB1, AB4, FN1,  
HH2, AB3,  
ML5, WCMH, IF5, HD37,  
HD6,  
F 103.11, B-E2, B-B8, B-  
F12  
anti-CD4, CD8

5 The examples described below illustrates embodiments and reflect the potential of the new method for detection and characterization of target cells, not previously known by persons with knowledge in the art. It was highly surprising that mixed cell populations could be incubated simultaneously, or

subsequently, with a number of different particle-bound antibodies, that for each antibody the binding of the antibody-particle complex to the target cells was specific and that the binding of different complexes could easily be visualized and distinguished in a fluorescence microscope with individual  
5 and/or several filters compatible with fluorescent emission spectra of the fluorescent microspheres, or by changing to conventional light microscopy to better identify binding of dyed non-fluorescent beads.

When several antibody-particle complexes are simultaneously incubated with  
10 target cells in a mixed cell suspension, one would easily have expected that the complexes could cluster or otherwise react with each other, forming complexes that unspecifically might bind to cells, that they for sterical or other reasons could block each others binding to target antigens, or that the fluorescence of the particles could be quenched, making it difficult to  
15 distinguish between the different types of particles. Surprisingly, however, by following the procedure according to the invention, no such problems are observed. The specificity of this approach is further demonstrated in experiments that included incubation of the target cells with one antibody-particle conjugate that would yield yellow fluorescence in the microscope,  
20 and thereafter with the same antibody coupled to a particle with a red fluorescence. In this case binding of the yellow antibody-particle conjugate was observed, whereas the binding of the second complex was completely blocked since the same antibody had been used for conjugation to both the yellow and the red particles.

25 In cases where the target cells in a mixed cell suspension are rare, such as tumor cells in peripheral blood and bone marrow, an enrichment procedure may be introduced before or in combination with the color/fluorescent-particle procedure. The enrichment can be obtained with different previously  
30 known approaches, including immunological procedures such as panning, column separation, or immunomagnetic positive or negative selection. If immunomagnetic selection is preferred, the same incubation step may include both the magnetizable and non-iron containing beads with the relevant cell binding antibody. After the enrichment step, the cell suspension containing  
35 the target cells can be examined and microscopically evaluated for fluorescent or dyed particle binding. Moreover, if immunomagnetic beads of

a size of for example at least 1  $\mu\text{m}$  are used for enrichment, such cell-bound beads can also be observed and used as an additional cell marker (Fig. 2).

The possibility of having a rapid, simple and reliable way of simultaneously mapping expression patterns of several relevant markers on cell populations, or at an individual cell level, opens new avenues in cell biology research and for routine diagnostic, staging and prognostic evaluation of a wide range of diseases, originating in all types of human and animal tissues. In many circumstances a rapid diagnosis is of great importance in the choice of therapeutic alternative. Examples of this includes surgical procedures to be chosen depending on whether e.g. a mammary, prostate or a brain tumor is malignant or not, whether a lymph node enlargement is caused by tumor cell infiltration or by an inflammatory reaction, on what type of cells that constitute thickening of synovial membranes in joints, what of type of cells that are present in surgical, needle, or fiberoptic biopsies from lesions in the skin, lung, liver, bone, ovaries or in the intestine and other tissues, and on what type of cells that might be present in pleural or ascitic effusions, in CSF, lymph, peripheral blood and bone marrow. At present, diagnosis of such cells are mainly based on morphological evaluations, and also on immunocytochemistry performed after preparation of tissue sections, cytopins or smears. Morphologically it can be difficult to determine the nature of the cells, and as previously mentioned immunocytochemistry can maximally detect the presence of two markers. With the new method, cells from the samples are dispersed e.g. in physiological saline or medium, incubated with relevant antibody-microsphere combinations for the necessary length of time, usually 30 minutes, and then examined microscopically. In the cell suspension, the bound particles can easily be recognized, permitting a suprisingly rapid and simple immunological fingerprinting or profile of the target cells. Because of this simplicity, the multiparameter characterization, the very short time frame needed to complete both the procedure and evaluation, the method represents a major contribution in the efforts to achieve rapid and reliable diagnosis of disease and obtain information of crucial importance for the further handling of patients.

To illustrate situations where such characterization is important the following teoretical examples are included:

In breast cancer it is known that the expression on the tumor cells of markers such as erbB2, EGF receptor, and IGF2 may be associated with increased proliferation and aggressiveness of the disease. In addition, the expression of other determinants such as EGP2, uPAR, VEGF, MUC1, MDR, Fas, and FasL can reflect characteristics that are important for the ability of the cells to metastasize, to induce angiogenesis, to resist chemotherapy, as well as for apoptosis of the tumor cells or the host T-cells. By using a combination of microspheres several of these parameters can be registered simultaneously in only one operation. Such studies can be performed on the biopsies from the primary tumor, on needle biopsies from solid metastases, and on samples from ascitic or pleural effusions, blood, and bone marrow.

In HIV-infected patients, the characterization of the different subsets of T lymphocytes is of vital importance. Examples of determinants that with the new method can be studied in addition to the most common T-cell markers are chemokine receptors and apoptosis-related molecules such as Fas and FasL.

In malignant melanoma the degree or lack of differentiation of the tumor cells may reflect the potential aggressiveness of the disease in the way that lesser differentiation is related to increased malignancy. In addition, several molecules are important for immunological response, including markers such as gp100, MAGE1, 2, 3, B7, Fas and FasL. Since such markers are important for the effect of immunotherapy and vaccination, comprehensive characterization of these as well as other melanoma-associated antigens are of great importance for the clinical management of the patients. Such characterization can readily be done with the new procedure.

Lymph node enlargement can reflect different types of reactive, infectious, or malignant conditions. Thus, it may be important to determine whether such lymph nodes contain tumor cells or not. If tumor cells are present, determination of the type of malignant cells can decisively influence the choice of therapy. One example is lymph node metastasis that could originate from either a small cell lung cancer (EGP2) or a malignant melanoma (HMW250000). With the appropriate choice of antibody-microsphere

conjugates this distinction can easily be made with the new approach within less than one hour.

## 5      EXAMPLES ON THE USE OF THE NEW PROCEDURE

### 1.      Specificity testing of antibody-conjugated fluorescent particles in human breast cancer cells.

10      MCF-7 human breast cancer cells were incubated with 1  $\mu$ m bright pink fluorescent microspheres coated with avidin, with or without biotin-conjugated MOC31 anti-EGP2 (anti-epithelial cell marker) antibody, and/or with immunomagnetic beads (4,5  $\mu$ m) coated with an anti-breast mucin (MUC1) antibody (BM7).

15      A suspension of MCF-7 cells incubated with fluorescent particles without bound MOC31 antibody was examined in a microscope. No fluorescent beads were attached to the tumor cells. Similar experiments with MOC31 biotin-avidin-conjugated fluorescent particles showed from 5 to 10 fluorescent  
20      particles bound to the surface of the tumor cells. In other experiments, MCF-7 cells were incubated with immunomagnetic beads coated with the BM7 antibody that bind to the tumor cells, followed by incubation with fluorescent particles with and without MOC31 antibody. It was found that the tumor cells with surface bound immunomagnetic beads showed binding also and only of  
25      MOC31-conjugated fluorescent particles. The two types of particles could easily be used in parallel, and the results showed no unspecific cell adherence of particles lacking targeting antibody.

### 30      2.      Effect of simultaneous or subsequent incubation with antibody-coated beads

35      Human SKBr3 breast cancer cells were incubated with various combinations of bright pink fluorescent latex microspheres conjugated with MOC31 antibody, with or without simultaneous or subsequent incubation with immunomagnetic beads coated either with MOC31 or with BM7 antibodies.

Bead sizes as in example 1. If both the fluorescent and immunomagnetic beads had the same targeting antibody and were incubated simultaneously, both types of beads were seen bound to the tumor cells. If either of these microspheres/beads were incubated first for 30 min and thereafter for another 30 min with the other antibody-conjugated particles, the binding of the second antibody-particle complex was completely blocked. The binding of each type of beads conjugated with different antibodies and incubated simultaneously showed the same binding pattern as that seen if each of them were studied in separate experiments.

3. Simultaneous binding of several types of microspheres/beads to the same target cells.

Breast cancer cells known to express a number of different antigens on their surface were incubated simultaneously with antibodies recognizing four different of these antigens. Each antibody had independently been conjugated to four types of microspheres/beads: 1) blue dyed latex microspheres (0.5 $\mu$ m), 2) bright pink fluorescent latex microspheres (1  $\mu$ m), 3) yellow fluorescent microspheres (1 $\mu$ m), and 4) immunomagnetic super-paramagnetic particles (4,5  $\mu$ m). The method according to the invention showed that the tumor cells did bind all the four different types of beads which could be clearly recognized by using a combination of fluorescence and light microscopy. The number of particles attached to each cell varied for each antibody-particle complex in accordance with the known expression pattern of the corresponding antigen. The antibodies recognized the following antigens: EGP2, MUC1, EGF receptor, and an independent epithelial marker recognized by the 595 antibody.

4. Binding of fluorescent microspheres on target cells after immunomagnetic enrichment.

MCF7 human tumor cells were added to mononuclear peripheral blood cells from healthy volunteers in a ratio of 1/1000 tumor cells to mononuclear cells. The cell suspension was incubated with MOC31 anti-epithelial antibody attached to bright pink fluorescent microspheres (1  $\mu$ m) through an

avidin/biotin bond and simultaneously with super-paramagnetic immunobeads (4,5  $\mu$ m) coated with the BM7 anti-MUC1 antibody. After 30 min of incubation magnetic selection of tumor cells with immunomagnetic beads bound to their surface was performed, and samples of the resulting cell suspension was examined microscopically. It was found that the remaining tumor cells with bead rosettes on their surface had also bound 5-10 fluorescent particles to the membrane, whereas a contaminating normal cells did not show binding of any of the particles/beads.

5. Binding of fluorescent cells to malignant ascitic cells

A suspension of ascitic cells drawn from a patient was brought to the laboratory without any information of the origin of the cells. The cell suspension was incubated with different antibody-coated fluorescent particles and paramagnetic immunobeads according to the invention. Particles coated with antibodies recognizing different marker antigens bound to the cells in the suspension, demonstrating the malignant and epithelial nature of the cells, thus confirming the diagnosis of ovarian cancer. In another case with ascitic fluid no cells with antibody-coated particles were seen, in agreement with the conclusion of the referring pathologist. In both cases, the method described in the invention provided the results on several different markers within 45 minutes, whereas the parallel morphological and immunocytochemical examination of a single marker was first completed more than 24 hours later.

6. Detection of cells in a pleural effusion

Without any prior knowledge of the underlying disease, incubation of the cell suspension with different anti-tumor antibodies coated onto fluorescent and immunomagnetic particles showed strong binding of all microspheres and immunobeads with anti-carcinoma and breast mucin (MUC1) antibodies. The diagnosis of the patient was breast cancer with pleural effusion. Microspheres conjugated with an anti-melanoma antibody did not bind. In conclusion, also in this case the cells in the clinical sample were successfully identified.

7. Needle aspirate from a thyroid tumor

- 5 The cell suspension obtained from needle aspirate was incubated with  
fluorescent microspheres with an antibody known to react with colorectal  
cancer cells, and simultaneously with immunomagnetic beads coated with the  
MOC31 anti-epithelial antibody. The fluorescent microspheres did not bind  
to any types of cells in the suspension, whereas the MOC31 immunobeads  
10 bound strongly to thyroid epithelial cells but not to the high number of  
macrophages present in the suspension.

The above examples demonstrate that the new method according to the  
invention shows considerably increased diagnostic strength and reliability in  
that a higher number of target cell antigenic determinants can be deleted in  
15 one operation, in a very short period of time compared to previously known  
methods.



## CLAIMS

1. Method to detect and phenotype target cells in cell suspensions by using particles coated with antibodies/ligands directed against antigenic determinants/receptors expressed on the target cells,  
5 **characterized in** that several types of particles, each particle instrumentally or visually separable by fluorescence, color and size, and each type of particle coated with a different antibody or ligand, are incubated simultaneously or subsequently with cell suspensions containing the target cells, in connection or not with a per se known enrichment procedure.
- 10 2. Method according to claim 1,  
**characterized in** that the particles used in the method are separable by a combination of fluorescence, color and/or size or a combination of fluorescent emission spectra, different colors or different sizes, preferably fluorescent emission spectra and/or size.
- 15 3. Method according to claim 1-2,  
**characterized in** that the size of the particles used in the method ranges from 0,01  $\mu\text{m}$  to 6  $\mu\text{m}$ , preferably from 0,5  $\mu\text{m}$  to 4,5  $\mu\text{m}$ .
4. Method according to claim 1-3,  
**characterized in** that it comprises the following steps;  
20 - adding several antibodies, ligands preferably 2-6, each antibody conjugated to specific separably particles, to the cell suspension containing the target cells, ratio 20:1 to 0.5:1, preferably 5:1 (number of particles/number of cells), limited by the size of the particles,  
- incubate under gentle rotation for 5-10 minutes to 2 hours, preferably 30  
25 minutes, at 0°C to 37°C, preferably 4°C,  
- evaluation of the target cell rosettes microscopially and/or by suitable visualizing or imaging devices.
5. Method according to claim 1-4,  
**characterized in** that the particles used in the method are coated with  
30 ligands/antibodies directed against adhesion molecules, carbohydrate antigens, glycolipids, growth factor receptors, melanoma antigens, sarcoma antigens, carcinoma markers, neuroblastoma antigens, glioma antigens, head and neck antigens, HLA-antigens, apoptosis-associated molecules, motility-

related antigens, proliferation-associated antigens, differentiation-associated antigens, drug resistant-related antigens, angiogenesis-associated antigens, chemokine receptors, invasion-related antigens, B- and T-cell CD antigens and various antigens, such as cathepsin D, neuroglandular antigen (CD63) and pan-human antigen.

6. Method according to claim 5, **characterized in** that the particles used in the method are coated with ligands/antibodies directed against the receptors/antigens listed in Table 1.

7. Method according to claims 5-6, **characterized in** that the particles used in the method are coated with antibodies such as MOC31 anti EGP2 (anti-epithelial cell marker) antibody, anti-breast mucin (MUC1) antibody (BM7), 595, anti-EGF receptor (425.3), anti-erbB2 and anti-HMW melanoma antigen (9.2.27).

8. Use of the method according to claim 1-7, wherein it is performed phenotyping of the target cells comprising profiling the antigenic determinants or receptors expressed on the cell membrane of the target cells.

9. Use according to claim 8, wherein the target cell characteristics of biologically informative markers of diagnostic, prognostic and therapeutic value are registered.

10. Use according to claim 9, wherein the target cells are malignant cells and/or HIV-infected T-lymphocytes.

11. Use according to claim 9, wherein the biologically informative markers are adhesion molecules, such as E-cadherin, CD44 variants, NCAM (CD56), growth factor receptors such as EGF, carcinoma marker such as EGP2, carbohydrate antigens such as Le<sup>y</sup>, CEA, 15-3 epitope, growth factor receptor such as EGFr, c-erbB2, IL-2 receptor, TNF receptor, melanoma antigens such as HMW 250000, gp 75/TRP-1, p95, MAG 1, 2, 3, sarcoma antigens such as TP 1 and TP 3 eptiopes, carcinoma markers such as EGP2, MUC1, MUC2 & 3, PSA, PSM, GA733.2, TAG72, 15-3 epitope, ovarian carcinoma DC 125 epitope, glioma antigens such as Mel-14 epitope, HLA antigens such as HLA class 1, HLA-DR, DQ, DP,  $\beta_2$ -microglobulin, apoptosis associated markers such as Fas, FasL, p75, motility related markers such as KAT-1, AMF, proliferation associated antigens such as gp120,

differentiation associated markers such as MUC 18, TA99, drug resistance markers such as MDR, MRP, angiogenesis associated antigens such as VEGF, bFGF, chemokine receptors such as CCR, CXCR, invasion-related markers such as uPAR, uPA, PAT-1, TIMP1 & 2 MMP9, stromelysins, B- and T-cell antigens such as CD2, CD10, CD4, CD8, CD19, CD20, CD22, CD37, and other antigens such as cathepsin D and par-human epitope.

12. Kit to perform the method according to claim 1-7, **characterized in** in that it comprises particles conjugated to antibodies/ligands according to claims 5-7.

1 / 2

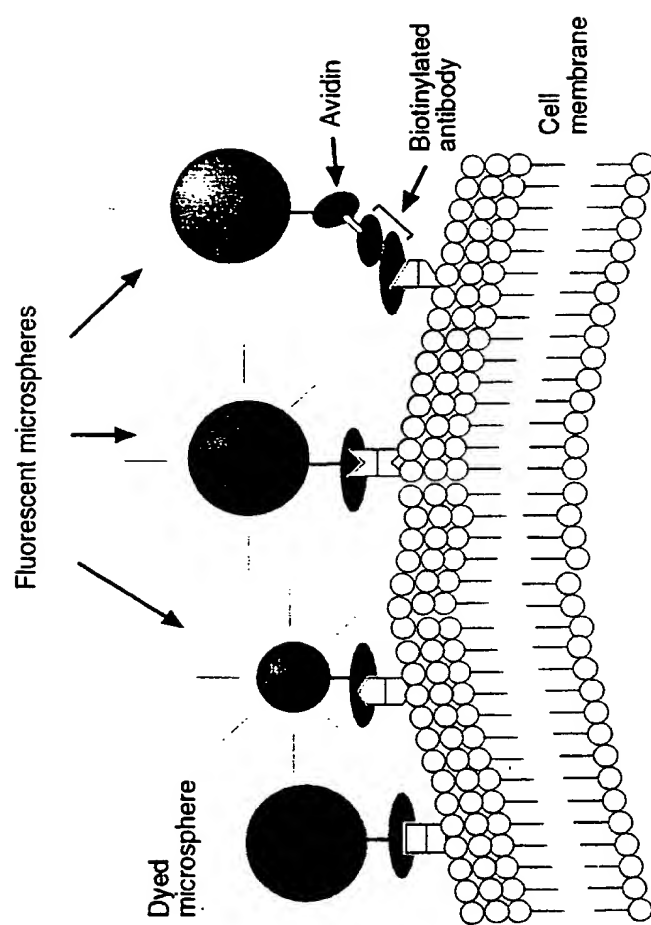


Fig. 1

2/2

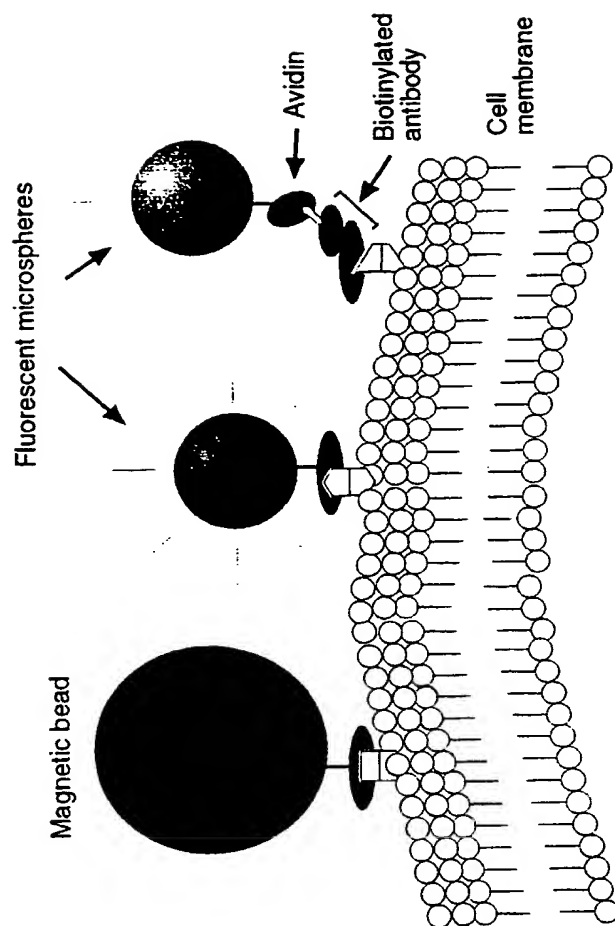


Fig. 2

# INTERNATIONAL SEARCH REPORT

1

International application No.

PCT/NO 97/00342

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/543, G01N 33/569  
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5340719 A (CONSTANCE M. HAJEK ET AL), 23 August 1994 (23.08.94), see the whole document  --	1-12
X	WO 9407142 A1 (SRI INTERNATIONAL), 31 March 1994 (31.03.94), see page 4-7, 12, 21-22, 36-38, 40  --	1-12
X	US 5326696 A (TSE W. CHANG), 5 July 1994 (05.07.94), see column 9-10 and claims  --	1-3,5-12
X	US 5405784 A (MICHEL VAN HOEGAERDEN), 11 April 1995 (11.04.95)  --	1-3,5-12

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document but published on or after the international filing date	"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

Date of mailing of the international search report

6 April 1998

16 -04- 1998

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2  
INTERNATIONAL SEARCH REPORT

International application No.  
PCT/NO 97/00342

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5219763 A (MICHEL VAN HOEGAERDEN), 15 June 1993 (15.06.93)  --	1-3,5-12
X	FR 2638849 A1 (CHEMUNEX), 11 May 1990 (11.05.90)  --	1-3,5-12
X	US 5194300 A (SAU W. CHEUNG), 16 March 1993 (16.03.93), see examples 4-8  --	1-12
X	US 5374531 A (BRUCE D. JENSEN), 20 December 1994 (20.12.94), see column 18 and claims  --	1-12
X	File WPI, Derwent accession no. 93-173192, TOYOBO KK: "Sensitivity detection of ligand-receptor reaction - by combining fluorescent fine particles with objective substance, passing mixt. through flow cytometer, counting number of agglomerates etc."; & JP,A,5107249, 930427, DW9321  --	1-3,5-12
X	DE 3811566 A1 (HITACHI, LTD.), 27 October 1988 (27.10.88), see column 8-10  --	1-12
X	US 5290707 A (SHEILA J. WOOD), 1 March 1994 (01.03.94), see column 6, line 49 - column 8  --	1-3,5,8,9,12
X	EP 0537827 A1 (EASTMAN KODAK COMPANY), 21 April 1993 (21.04.93), see example 6 and claims  --	1-3
A	BIOTECHNOLOGY, Volume 3, April 1985, K. A. Muirhead et al, "Flow cytometry: present and future", page 337 - page 356, see pages 341-345 and pages 349-351  --	1-12





# INTERNATIONAL SEARCH REPORT

Information on patent family members

02/03/98

International application No.

PCT/NO 97/00342

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US 5340719 A	23/08/94	AU 652599 B AU 9091091 A CA 2096502 A CN 1062212 A EP 0558639 A HU 67053 A IL 100124 A JP 6503171 T MX 9102194 A US 5348859 A US 5554505 A WO 9209878 A ZA 9109253 A	01/09/94 25/06/92 24/05/92 24/06/92 08/09/93 30/01/95 31/12/95 07/04/94 30/06/94 20/09/94 10/09/96 11/06/92 24/05/93
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Information on patent family members

02/03/98

International application No.

PCT/NO 97/00342

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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US 5290707 A	01/03/94	NONE	
EP 0537827 A1	21/04/93	AU 2115092 A CA 2076592 A JP 5209887 A	22/04/93 09/04/93 20/08/93
WO 9631777 A1	10/10/96	AU 5137696 A AU PN214095 D EP 0819251 A	23/10/96 00/00/00 21/01/98

# PATENT COOPERATION TREATY

## PCT

### INTERNATIONAL PRELIMINARY EXAMINATION REPORT



(PCT Article 36 and Rule 70)

Applicant's or agent's file reference Fod 5/he		See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416) <b>FOR FURTHER ACTION</b>	
International application No. PCT/NO97/00342	International filing date (day/month/year) 16/12/1997	Priority date (day/month/year) 20/12/1996	
International Patent Classification (IPC) or national classification and IPC G01N33/543			
Applicant FODSTAD, Oystein et al.			

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 7 sheets, including this cover sheet.
  - ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 22 sheets.

3. This report contains indications relating to the following items:
  - I ☒ Basis of the report
  - II ☐ Priority
  - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
  - IV ☐ Lack of unity of invention
  - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
  - VI ☐ Certain documents cited
  - VII ☐ Certain defects in the international application
  - VIII ☒ Certain observations on the international application

Date of submission of the demand 28/05/1998	Date of completion of this report 18.03.99
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. (+49-89) 2399-0 Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Authorized officer Knudsen, H Telephone No. (+49-89) 2399 8696 

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/NO97/00342

**I. Basis of the report**

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.)*:

**Description, pages:**

20	as originally filed	
1-19	with telefax of	10/02/1999

**Claims, No.:**

1-14	with telefax of	10/02/1999
------	-----------------	------------

**Drawings, sheets:**

1/2,2/2	as originally filed
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2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

**see separate sheet**

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NO97/00342

## V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

### 1. Statement

Novelty (N)	Yes:	Claims	1-13
	No:	Claims	14
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-14
Industrial applicability (IA)	Yes:	Claims	1-14
	No:	Claims	

### 2. Citations and explanations

**see separate sheet**

## VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

**see separate sheet**

## **ITEM I:**

1. The use of disclaimers in claims is generally allowed only when the claimed subject-matter cannot be defined in positive terms (cf PCT Guidelines C-III 4.12). In the present case, the applicant argues that the specific reaction conditions mentioned in claim 1 distinguish the claimed method from the prior art. Apparently, the disclaimer is therefore not needed for defining the claimed subject-matter and its use therefore contravenes Article 34(2)(b) PCT. This report is therefore drafted as if claim 1 did not contain the disclaimer.
2. The original application does not contain any basis for the mention of 25°C as reaction temperature. This part of present claim 1 therefore goes beyond the content of the original application. In the letter of reply, the applicant has indicated that the subject-matter of original claim 4 is introduced into claim 1 and present claim 1 is therefore examined as if the temperature were "37°C".
3. Finally, the term "PAI-1" in claim 13 does not appear to be mentioned in the original application and claim 13 is therefore examined as if the said term would be PAI as in original Table I.

## **ITEM V:**

### **NOVELTY:**

4. US-A-5,340,719 (D1) discloses an optical screening method, in which the cells are combined with one or more different sets of microspheres. The microspheres can differ in colour or size (cf claims 18 and 20). Each set of microspheres contain a different reactant, which may be an antibody, binding to different antigens present on the surface of the tested cells. The bound cells may be observed in a smear. The cells are distinguished by their morphology and the different colours and/or sizes of the microspheres bound to the cells. The microspheres have a size between 0.7 and 3.06  $\mu\text{m}$ . The assay concerns mainly the profiling of white blood cells (WBC) and it is indicated in D1 that whole blood normally contain from 4-11000 WBC per  $\mu\text{l}$  of whole blood and in the exemplified assay (column 11) 100  $\mu\text{l}$  whole blood is mixed with 40  $\mu\text{l}$  of microspheres containing  $2 \times 10^7$  particles/ml.

This gives a ratio of  $8 \times 10^5$  particles per  $4-11 \times 10^5$  WBC. This ratio clearly falls within the ratio mentioned in claim 1. The mixture is incubated for 10-30 minutes. However, there is no exact indication of the temperature.

Claims 1, 3-4 and 9-11 are therefore distinguished from D1 only in that the reaction is carried out at temperatures between 0 and 37°C.

The IPEA is of the opinion that the reaction in D1 is carried out at room temperature in the absence of other information, but does not wish to raise a novelty objection in the absence of this information.

WO 94/07142 (D2) discloses an assay for the determination of the presence and relative abundance of T-lymphocyte subpopulations (cf p.40). The assay employs six different antibodies that are all specific for a different CD-antigen. Each of the antibodies is attached to a different particle. Three are attached to three groups of superparamagnetic particles of different size and three to different up-converting phosphor particle labels emitting light of different wavelengths. The binding of the particles to the cells in a blood sample is used to evaluate the types of T-lymphocytes present in the sample and can be used to measure a patient's response to chemotherapy. The phosphor-converting particles are smaller than 3  $\mu\text{m}$ . Moreover, D2 discloses that the colour of the superparamagnetic particles can be used for differentiation as well (cf p.42, line 13) and be used for distinguishing the particles. However, D2 does not disclose any exact ratio of particles to cells.

As regards, the other X-documents cited in the International Search Report, they do not appear to disclose a specific ratio of particles to target cells either.

Claims 1-13 are therefore considered novel.

5. US-A-5,405,784 (D3) discloses an assay employing differently coloured particles. The fixation of lectins with different specificity to differently coloured particles is disclosed in Example 6 and the immobilization of anti-IgG and anti-IgM antibodies to particles with different colours is disclosed in Example 3. Claim 14 therefore lacks novelty over D3.

**INVENTIVE STEP:**

6. As indicated above, the method of claims 1, 3-4 and 9-11 is distinguished from D1 only in the reaction temperature. The IPEA is of the opinion that a temperature between 0 and 37°C is the only temperature that the skilled person would consider when carrying out the claimed assay. The said claims are therefore not considered inventive.
7. The use of an incubation temperature of 4°C is not disclosed in the prior art, but would appear to be obvious for the skilled person who wishes to avoid internalization of the antibodies and changes of the cell surface antigens. Claim 2 is therefore not inventive.
8. "BIOTECHNOLOGY, vol.3, pages 337-356, (1985)" (D4) discusses the possibility of carrying out multiparametric studies of malignant cells, but indicates that solid tumour cells may be difficult to examine due to cell surface alteration during dispersal (cf paragraph bridging pages 348 and 349). In the light of D4, the skilled person would therefore consider the possibility of analysing malignant cell surface antigens once the problems with dispersal of the sample is solved. The claimed method does not require any specific dispersal of the sample and it therefore appears that the dispersal problems have been solved since the publication of D4 (ie 1985). Consequently, it would have been obvious for the skilled person to test solid tumour surface antigens in the same way as the malignant lymphatic cells are analysed in D1 and D2.

The applicant argues that the claimed method is distinguished from D1 and D2 in that the claimed method is designed to characterize individual live cells present in low numbers and therefore must contain few and mild steps which do not result in clumping and agglutination. The reaction conditions used in the method of claim 1 leads to this high specificity and lack of alteration of antigens.

The IPEA is of the opinion that the requirements mentioned by the applicant are not reflected in the claims (ie there is no mention of individual live cells and it is not clear how aggregation can be avoided when practically the same conditions are used as in D1, which according to the applicant leads to aggregation.



Moreover, the number of treatment steps appear to include those during dispersal of the cell sample. However, this stage is not mentioned in the claims and it is thus not clear whether this stage is of importance for avoiding aggregation. Finally the passage referred to in the letter of reply (ie column 12, lines 18-39) is not found in the original application.

The IPEA is therefore not convinced by the arguments conveyed by the applicant and considers claims 5-8 and 12-13 not to be inventive.

**INDUSTRIAL APPLICABILITY:**

9. The claimed method is carried out in-vitro on samples. The claimed subject-matter is therefore considered industrially applicable.

**ITEM VIII:**

10. It is clear from the description (p.7, l.34-35) that the ratio of particles to cells mentioned in the claims refer to the number of target cells. However, the claims which do not contain this feature lack clarity, because the number of cells could also refer to the total number of cells in the cell suspension.
11. The features following "optionally" and "such as" in claim 1 are merely optional features which do not have any limiting effect on the scope of the claim and should be incorporated into a separate dependent claim for reasons of clarity.
12. Only one of the prior art documents disclose the ratio of particles to target cells and it therefore appears that the ratio defined in claim 1 is unusual in the art and should not be used for defining the scope of the claim (cf PCT Guidelines C-IV 4.7a).

1 510 Recl PCT/PTO 18 JUN 1999

**Method for characterization of abnormal cells**

The present invention relates to a new method for identification and characterization of eukaryotic cells.

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In several diseases such as primary and secondary malignancies, allergic, autoimmune, inflammatory, proliferative, infectious, and destructive disorders, or diseases for which the underlying mechanisms are unclear, it would be of utmost importance to be able to determine as many as possible characteristics of cells involved in the pathologic processes. An exact determination of a number of different markers on such cells would significantly improve diagnosis, prognostication and the choice of subsequent therapy. If such a procedure is simple and rapid, it would be easy to diagnose pathological conditions at an early stage of the disease, thereby increasing the probability of selecting the best therapeutic alternative at a time when the treatment may be most effective. Moreover, in some situations it is of crucial importance to make an immediate and correct diagnosis, such as to distinguish between a benign and a malignant tumor, to guide in the surgeons' selection of a proper operative procedure.

Presently, the following diagnostic methods related to the above mentioned pathological conditions are at hand: Conventional morphological examination of tissue sections, cell cytopspins or smears, and immunological methods including immunocytochemistry, flowcytometry, and immunofluorescence microscopy. In addition, peroperative morphological evaluation of biopsied tissue specimens are performed on frozen sections.

With the non-immunological methods, the diagnosis can only provide distinction between normal and pathological cells based on morphological criteria.

Immunological methods such as immunocytochemistry and flowcytometry represent valuable diagnostic tools, although they suffer from several important limitations. With both methods, heterogenous cell populations are exposed to antibodies or other ligands for their binding to target cells. For flowcytometry studies live or fixed cells may be incubated to allow for

*Replaced by Article 34*

fluorescence-labeled antibodies to bind to relevant membrane or intracellular antigens, before the cell suspension is analyzed in the instrument. Immunocytochemistry requires preparation of tissue sections, cytopins or smears, fixation and immunostaining of the cells before evaluation in a microscope. Visualization of bound antibodies is obtained indirectly through one or several steps ending with an enzyme/substrate color reaction, allowing the stained cells to be observed in a microscope. The multi-step procedure can not be completed on the day of cell sampling. Moreover, usually thorough evaluation by an experienced pathologist is needed for obtaining reliable results. For example, if the abnormal cells are being identified in a mixed cell population, and the ratio of pathological to normal cells is low, such as malignant cells in samples of bone marrow or peripheral blood, an excessive amount of work performed by a skilled pathologist may be needed for cell identification. Another problem is related to the fact that there are very few antibodies recognizing antigens that are selectively and consistently expressed in all target cells. If the objective is to identify tumor cells in blood and bone marrow, antibodies directed against "tissue-specific" markers, such as cytokeratins found in epithelial cells, are commonly used. However, as it is known that some normal cells may also express cytokeratins and that not all malignant epithelial cells do, there is a risk of both false positive and negative results.

Fluorescence-labeled antibodies can be used to detect target cells either by fluorescence microscopy or by flow cytometry. The former procedure can successfully be employed to demonstrate binding of a single antibody, although the use of morphological criteria as an additional way of distinguishing between normal and pathological cells is very limited. Moreover, the fluorescence usually fades and disappears rapidly under examination in the microscope. Thus, it is required that the fluorescence cells are studied and assessed microscopically within a short timeframe after binding of the antibody. Flowcytometric analysis requires the presence of a high number of target cells to provide reliable results. Moreover, the procedure does not provide any possibilities for morphological studies or for distinguishing between fluorescent target and non-target cells. Furthermore, several mentioned methods have the disadvantage that cells are lost in the methodological steps.

Improved possibilities for detecting target cells have recently been described (WO94/07138, WO94/07139, WO95/24648). In these procedures, antibodies bound to super-paramagnetic particles are used for detection and selection of the cells to be identified. One limitation of these methods is that it can be difficult to prove directly the pathological nature of cells with bound particles on the surface. One advantage compared to the other described methods is, however, the simplicity of the procedure and that results can be obtained within a very short timeframe.

To further confirm the pathologic nature of stained, fluorescent or immunobead-binding cells it is highly warranted to characterize the target cells for more than one marker. The aim is to obtain important biological information, and information of crucial diagnostic and prognostic significance. If the number of target cells is high, flow cytometry may be used to study in parallel the binding of two different cell-bound antibodies. However, this method lacks the possibility to examine individual cells and cell morphology, and actually to identify fluorescent cells as the real target cells. Immunocytochemistry does allow for a maximum of two markers to be studied in parallel, one with conventional enzymatic visualization of bound antibody and one with a silver/gold enhancement procedure. Both multi-step procedures are relatively complicated, time-consuming and requires either expensive equipment and/or special expertise in the respective areas.

With the immunomagnetic method, further characterization of whole cells may be obtained by preparing cytopins of the magnetically selected cells and thereafter performing immunostaining as for conventional immunocytochemistry. Therefore, the same limitations as described for immunocytochemistry apply, and furthermore because the target cells have beads attached to their surface it may be difficult to get the cells to stick to the slides used for conventional cytopin preparation.

In conclusion, the existing methods provide possibilities for studying a maximum of two independent markers, and inherent to the described methods several important problems and limitations are present. It was therefore highly warranted to develop a method that much more simply, rapidly, and

reliably could help identifying and characterizing target pathological cells. The complexity and heterogeneity of cell biology makes it also warranted to be able to examine expression of several independent biological markers on the same cells. Such biological information would be of vital diagnostic and prognostic significance that can aid in the choice of therapeutic alternative. When several markers are examined in parallel it would be possible to obtain a more reliable confirmation of the pathological nature of the target cells, thereby improving the diagnostic reliability and help excluding false positives as well as negatives. Importantly, multi-parameter characterization could include markers of cell proliferation, cell death (such as apoptosis), adhesion, motility, invasion, antigenicity, inflammation, cell destruction, auto-immune mechanisms, angiogenesis, disease aggressiveness, tumor metastasis, and inhibitors of all these functions. Furthermore, if several markers can be examined also at an individual cell level, it would be possible to study cell heterogeneity and identifying subsets of cells with specific biological properties. In some diseases it would also be important to study pathological cells obtained from different sites in the same patient in order to determine whether cell characteristics could vary from one site to another, thus providing additional biologically and prognostically important information. Altogether, the impact of obtaining information of the type here described for clinical evaluation and treatment of patients can hardly be overestimated.

These objects are obtained in the present invention as characterized by the enclosed claims.

We here introduce a new concept in characterization of intact target cells in cell suspensions, making a direct microscopical identification of more than two membrane-associated markers possible. With this method several cell membrane markers of the origin, biology and potential fate of target cells can be studied in the same operation. The procedure is very simple and can be completed within a very short timeframe without the need for advanced and expensive instrumentation. With the method, target cells can with a minimum of handling steps, without any cell loss, be studied microscopically for the expression of several independent marker molecules, even at the individual cell level. Thus, in addition to obtaining an overall picture of biological

parameters present in the target cell population, such a procedure also allows for examining cell to cell variation in the expression of marker molecules, providing information with vital biological and medical implications.

5 Briefly, the method can be performed as here described: Dyed or fluorescent microspheres (beads, particles) conjugated with antibodies or ligands that can bind to cell membrane determinants to be studied are added to the cell suspension and incubated under gentle rotation. Thereafter, samples of the cell suspension are examined in a fluorescence microscope for cells with  
10 surface-bound microspheres of different light or fluorescent colors. The extent and variation in cell binding of the different microspheres can be assessed and quantitated. The assessment of cell-bound particles may, if warranted, be performed by an automated procedure.

15 In the following the present invention is described in greater detail with the examples, which by no means are intended to restrict the invention, and figures in which:

Fig. 1 illustrates the binding of four types of microspheres to four different  
20 antigenic determinants expressed on the membrane of a target cell. The binding is in this case mediated through four different antibodies, each recognizing one of the said four antigens, in that the antibodies had first been conjugated to the respective microspheres, either directly through a chemical bond (Fig. 1, the three examples to the left), or indirectly where the beads  
25 had been pre-coated with avidin before conjugation to a biotinylated antibody (Fig. 1, right example). As illustrated in Fig.1, one antibody was bound to a blue dyed microsphere, one antibody to a small and another to a larger red fluorescent microsphere, whereas the fourth antibody had been biotin-avidin conjugated to a green fluorescent microsphere.

30 Fig. 2 illustrates how the invention can be used to characterize two or more cell membrane determinants in a situation where the target cells in a mixed cell suspension are rare, thus warranting an enrichment procedure before evaluation of the sample. In the illustrated case, antibody coated  
35 superparamagnetic beads are bound to the cell membrane together with red and green fluorescent microspheres conjugated either directly or through an

avidin-biotin binding to separate antibodies. In such a situation, immunomagnetic enrichment can be obtained by using a strong magnet that will attract cells with bound magnetic beads. The enriched cell suspension is thereafter examined in a microscope in which the binding of the fluorescent microspheres to target cells with bound magnetic beads can be observed.

The visually or instrumentally different dyed or fluorescent particles, which can be of similar or different sizes, used in the invention are conjugated to ligands such as antibodies, or fragment of antibodies, lectins and growth factors, that can bind to specific molecules expressed on membranes of abnormal cells, so that the bound particles can be identified microscopically. Examples include the use of polystyrene latex fluorescent microspheres of various colors that can be observed in a fluorescence microscope, and dyed non-fluorescent particles, such as red, yellow, green, black and blue, that can be detected by light microscopy. Antibodies conjugated to the microspheres include all those recognizing antigens, receptors, and other determinants expressed on membranes of abnormal cells, and on normal cells, see below. By combining different antibody-particle conjugates relevant for the cells to be studied, a finger-print of cell characteristics can be obtained rapidly and directly in the cell suspension. Such antigenic finger-prints would be highly valuable in evaluating important biological characteristics of cells, see above, cell populations or sub-populations. The simplicity and speed by which the method can provide such information is surprising and constitutes a key element of the invention.

Antibody-conjugated fluorescent and dyed particles have been used in various types of immunoassays to determine, e.g. the presence of free antigens, proteins, viruses and bacteria in biological fluids. With intact eukaryotic cells, fluorescent microspheres conjugated to antibodies have been used to study in each case a single molecule expressed in a specific type of normal cells, such as monocytes, lymphocytes, hepatocytes and fibroblasts. The purpose of these studies have been such as examination of the motility of membrane markers in macrophages or metabolic parameters in hepatocytes. There is no report found in the literature on attempts to study abnormal cells, such as malignant and benign neoplastic cells, and abnormal cells found in various infectious, reactive, autoimmune, inflammatory and

proliferative disorders. Furthermore, combination of several antibodies conjugated to different dyed or fluorescent microspheres on the same cell population, or on individual cells, are not described. Also, such procedures have not been employed to study, for biological or diagnostic purposes, sub-  
5 populations of target cells in a mixed population of cells.

The particles to be used can be fluorescent polystyrene latex microspheres or non-fluorescent particles of different colors. The size of the microspheres can be between 0.01  $\mu\text{m}$  and 6  $\mu\text{m}$ . The particles should provide possibilities for  
10 conjugating antibodies or other ligands to their surface. This may be obtained directly, such as through chemical groups like carboxyl, amino or other groups, or indirectly by binding antibodies to microspheres previously coated with proteins such as avidin, streptavidin, protein A, or with antibodies that can react with a second antibody. The size of the microspheres may be  
15 chosen to fit the size of the cells and the purpose of investigation, such that it would facilitate identification of different bound antibody-microsphere conjugates. It is considered that a particle size of e.g. 1  $\mu\text{m}$  makes identification of a relatively low number of bound particles easy, whereas a smaller size may possibly be advantageous if a marker protein expressed at  
20 high density is to be studied. Another important feature of the invention is that it can be applied both when a very low or a very high number of cells are to be examined. It is also important that the fluorescent microspheres can retain their fluorescence strength for a considerable length of time.

25 The antibodies recognizing the relevant membrane marker antigens or receptors could either be whole IgG of any isotype, IgM antibodies or any fragments of such antibodies, including also recombinant antibodies or antibody fragments. The novel method includes binding of the said fluorescent or dyed microspheres to target cells in a suspension with a low  
30 number of non-target cells, and in other cases where the number of target cells is low compared to non-target cells. The cell suspension is incubated with several antibodies, preferably 2-6, each conjugated to different microspheres, of the same or different sizes, of a specific dye or fluorescent color. The ratio between the number of particles and the number of target  
35 cells ranges from 20:1 to 0.5:1, preferably 5:1, limited by the size of the particles. The cell suspension should be incubated with antibody-coated



beads for 5-10 minutes to 2 hours, preferably for 30 minutes, at 0°-37°C, preferably at 4°C under gentle rotation. After incubation, samples of the cell suspension is taken for evaluation in a fluorescence microscope or in other visualizing or imaging devices in which fluorescent particles and dyed particles can be observed. Microspheres that are bound to cells can then be visualized, and the number of cells with the different types of particles attached to their surface can be assessed, with or without enumeration also of the number of beads attached to the cells. Since it is possible to use a combination of several antibody-coated microspheres, fluorescence filters suited to study different fluorescence emission spectra may be used. The method also provides possibilities for semi-automatic, video, and computer image analysis of the presence of dyed or fluorescent particles bound to the cells.

The antibodies could be of murine, rat, rabbit or human origin and may preferably recognize antigens present on target cells and not on normal cells in mixed cell suspensions. A list of antibodies/ligands includes, but are not limited to, those directed against groups of antigenic determinants, for example CD56/NCAM antigen, pan-epithelial EGP2/cluster2 antigen, breast mucin (MUC1) and other mucin epitopes, HMW and other melanoma-associated antigens such as gp100, MAGE 1,2 and 3, and MUC18, 80kD sarcoma associated antigen, erbB2, receptors for growth factor such as EGF, TGF, PDGF, bFCF, VEGF, IGF1, and IGF2, laminin, laminin5, uPA, uPAr, PAI, TIMP1 and 2, stromelysin, and other invasion related molecules, CEA, PSA, PSM, NSE, c-Met, CD44 and variants, ICAM-1, integrins, cadherins, catenins and other cell adhesion-associated molecules, drug resistance markers such as MDR and MRP, apoptosis-related molecules such as Fas and FasL, markers of cell proliferation, motility, differentiation, metastasis, angiogenesis, signal transduction, and inflammation-related membrane molecules, oncogene products, and chemokine receptors such as CCR 1-5, CXCR 1-4, and Duffy antigen, and all types of hematopoietic and lymphatic cell markers categorized in the CD system. Table 1 lists groups of membrane determinants that can be targeted and a number of examples within each group is also presented.

Table 1.

Antigens/receptors and corresponding antibodies/ligands

ANTIGENS/RECEPTORS	EXAMPLES OF ANTIBODIES/LIGANDS
<b>Adhesion molecules</b>	
Integrins	Pierce 36114, BTC 21/22, M-Kiol 2 BTC 41/42, Calbiochem 407277-84
ICAM-1 (CD54)	C57-60, CL 203.4
VCAM-1	Genzyme 2137-01
NCAM (CD56)	MOC 1
HCAM	BCA 9
LCAM	BM 1441 892
CD44	BM 1441 272, 25.32
CD44 variants	11.24, 11.31
ELAM-1	Genzyme 2138-01
E-selectin	BBA 8
P-selectin	BTC 71/72
LFA-3 (CD58)	TS 2/9
MACAM-1	NKI-M9
E-cadherin	BTC 111, 6F9
P-cadherin	NCC-CAD-299
Tenascin	BM 1452 193
Thrombospondin receptor (CD36)	BM 1441 264
VLA-2	A1.43
<b>Carbohydrate antigens</b>	
T-antigen	HH8, HT-8, Lectins
Tn-antigen	TKH6, BaGs2, Lectins
Sialyl Tn	TKH-2
Galβ1-4GlcNac (nL4, 6, 8)	1B2, Lectins
Gastrointestinal cancer associated	

antigen (M.200kD)	CA 19-9
Le <sup>y</sup>	MLuC1, BR96, BR64
di-Le <sup>x</sup> , tri-Le <sup>x</sup>	B3
CA15-3 epitope	CA15-3
CEA	I-9, I-14, I-27, II-10, I-46,
Lacto-N-fucopentannose III (CD15)	PM-81

**Glycolipids**

GD <sub>3</sub>	ME 36.1, R24
GD <sub>2</sub>	ME 36.1, 3F8
Gb <sub>3</sub>	38-13
GM <sub>3</sub>	M2590
GM <sub>2</sub>	MKI-8, MKI-16
FucGM <sub>1</sub>	1D7, F12

**Growth factor receptors**

EGF receptor	425.3, 2.E9, 225
c-erbB-2 (HER2)	BM 1378 988, 800 E6
PDGF $\alpha$ receptor	Genzyme 1264-00
PDGF $\beta$ receptor	Sigma P 7679
Transferrin receptor	OKT 9, D65.30
NGF receptor	BM 1198 637
IL-2 receptor (CD25)	BM 1295 802, Bm 1361 937
c-kit	Bm 428 616, 14 A3, ID9.3D6
TNF-receptor	Genzyme 1995-01, PAL- M1
NGF receptor	

**Melanoma antigens**

High molecular weight antigen (HMW 250.000)	9.2.27, NrML5, 225.28
Mw105 melanoma-associated glycoprotein	ME20
100 kDa antigen (melanoma/carcinoma)	376.96
gp 113	MUC 18

p95-100	PAL-M2
gp75/TRP-1	15.75, TA99
gp 100-107	NKI-beteb
MAA	K9.2
M125kD (gp125)	Mab 436
MAGE 1, 2, 3	anti-MAGE 1, 2, 3
Tyrosinase	anti-tyrosinase
<b>Sarcoma antigens</b>	
TP-1 and TP-3 epitope	TP-1, TP-3
M.200kD	29-13, 29.2
M.160.kD	35-16, 30-40
<b>Carcinoma markers</b>	
EGP-2 (cluster 2 epithelial antigen)	MOC-31, NrLu10
MUC-1 antigens (such as DF3-epitope (gp290kD)	BM7, DF3, BCP-7 to -10
MUC-2 and MUC-3	PMH1
LUBCRU-G7 epitope (gp 230kD)	LUBCRU-G7
Prostate specific antigen	BM 1276 972
Prostate cancer antigen	E4-SF
Prostate high molecular antigen	PD41
M.> 400kD	
Polymorphic epithelial mucins	BM-2, BM-7, 12-H-12
Prostate specific membrane antigen (Cyt-356)	7E11-C5
Human milk fat globulin	Immunotech HMFG-1, 27.1
42kD breast carcinoma epitope	B/9189
M <sub>w</sub> > 10 <sup>6</sup> mucin	TAG-72, CC-49, CC-83
Ovarian carcinoma OC125 epitope (m. 750 kD)	OC125, OVX1
Pancreatic HMW glycoprotein	DU-PAN-2
Colon antigen Co-17-1A (M.37000)	17-1A
Ga 733.2	GA733, KS1.4

TAG 72	B72.3, CC-49, CC-83
Pancreatic cancer-associated	MUSE 11
Pancarcinoma	CC49
Prostate adenocarcinoma-antigen	PD 41
M <sub>w</sub> 150-130kD adenocarcinoma of	AF-10
M <sub>w</sub> 92kD bladder carcinoma	3G2-C6
M <sub>w</sub> 600kD bladder carcinoma	C3
Bladder carcinoma antigen	AN43, BB369
Hepatocellular carcinoma antigen	KM-2
M.900kD	
M <sub>w</sub> 48kD colorectal carcinoma	D612
Colon specific antigen	Mu-1, Mu-2
Lung carcinoma antigen M. 350-420kD	DF-L1, DF-L2
Colon cancer-associated	C242, NCRC37
Bladder carcinoma antigens	T16, T43, T138
<b>Neuroblastoma antigen</b>	
Neuroblastoma-associated, such as UJ13A epitope	UJ13A
<b>Glioma antigens</b>	
Mel-14 epitope	Mel-14
HMW 250kD	9.2.27
<b>Head and neck cancer antigens</b>	
M.18-22kD antigen	M48
<b>HLA-antigens</b>	
HLA Class 1	TP25.99
HLA-A	VF19LL67
HLA-B	H2-149.1
HLA-A2	KS1
HLA-ABC	W6.32
HLA-DR, DQ, DP	Q 5/13, B 8.11.2
β <sub>2</sub> -microglobulin	NAMB-1

**Apoptosis associated molecules**

Fas (CD95/APO-1)

Apo 1

FasL

Anti-FasL

P75

NGF

**Various**

cathepsin D

CIS-Diagnostici, Italy

neuroglandular antigen (CD63)

ME91, NKI-C3, LS62

pan-human cell antigen

pan-H

**Motility related antigens**

anti-KAI-1, anti-AMF

**Proliferation-associated markers**

anti-gp120, anti-Ki-67

**Differentiation-associated markers**

MUC 18, TA99

**Drug resistant-related markers**

C 219, MRK 16, anti-MRP

**Angiogenesis-associated markers**

anti-VEGF, anti-bFGF

**Chemokine receptors markers**

anti-CCR, anti-CXCR

**Invasion-related antigens**

Antibodies to:

PAI, MMP1, MMP9,

TIMP1, TIMP2,

laminin V, stromelysin,

uPAR, uPA

**B- and T-cell CD antigens**

HH1, AB1, AB4, FN1,

HH2, AB3,

ML5, WCMH, IF5, HD37,

HD6,

F 103.11, B-E2, B-B8, B-

F12

anti-CD4, CD8

5 The examples described below illustrates embodiments and reflect the potential of the new method for detection and characterization of target cells, not previously known by persons with knowledge in the art. It was highly surprising that mixed cell populations could be incubated simultaneously, or

subsequently, with a number of different particle-bound antibodies, that for each antibody the binding of the antibody-particle complex to the target cells was specific and that the binding of different complexes could easily be visualized and distinguished in a fluorescence microscope with individual  
5 and/or several filters compatible with fluorescent emission spectra of the fluorescent microspheres, or by changing to conventional light microscopy to better identify binding of dyed non-fluorescent beads.

When several antibody-particle complexes are simultaneously incubated with  
10 target cells in a mixed cell suspension, one would easily have expected that the complexes could cluster or otherwise react with each other, forming complexes that unspecifically might bind to cells, that they for sterical or other reasons could block each others binding to target antigens, or that the fluorescence of the particles could be quenched, making it difficult to  
15 distinguish between the different types of particles. Surprisingly, however, by following the procedure according to the invention, no such problems are observed. The specificity of this approach is further demonstrated in experiments that included incubation of the target cells with one antibody-particle conjugate that would yield yellow fluorescence in the microscope,  
20 and thereafter with the same antibody coupled to a particle with a red fluorescence. In this case binding of the yellow antibody-particle conjugate was observed, whereas the binding of the second complex was completely blocked since the same antibody had been used for conjugation to both the yellow and the red particles.

25 In cases where the target cells in a mixed cell suspension are rare, such as tumor cells in peripheral blood and bone marrow, an enrichment procedure may be introduced before or in combination with the color/fluorescent-particle procedure. The enrichment can be obtained with different previously  
30 known approaches, including immunological procedures such as panning, column separation, or immunomagnetic positive or negative selection. If immunomagnetic selection is preferred, the same incubation step may include both the magnetizable and non-iron containing beads with the relevant cell binding antibody. After the enrichment step, the cell suspension containing  
35 the target cells can be examined and microscopically evaluated for fluorescent or dyed particle binding. Moreover, if immunomagnetic beads of

a size of for example at least 1  $\mu\text{m}$  are used for enrichment, such cell-bound beads can also be observed and used as an additional cell marker (Fig. 2).

5 The possibility of having a rapid, simple and reliable way of simultaneously mapping expression patterns of several relevant markers on cell populations, or at an individual cell level, opens new avenues in cell biology research and for routine diagnostic, staging and prognostic evaluation of a wide range of diseases, originating in all types of human and animal tissues. In many circumstances a rapid diagnosis is of great importance in the choice of  
10 therapeutic alternative. Examples of this includes surgical procedures to be chosen depending on whether e.g. a mammary, prostate or a brain tumor is malignant or not, whether a lymph node enlargement is caused by tumor cell infiltration or by an inflammatory reaction, on what type of cells that constitute thickening of synovial membranes in joints, what of type of cells  
15 that are present in surgical, needle, or fiberoptic biopsies from lesions in the skin, lung, liver, bone, ovaries or in the intestine and other tissues, and on what type of cells that might be present in pleural or ascitic effusions, in CSF, lymph, peripheral blood and bone marrow. At present, diagnosis of such cells are mainly based on morphological evaluations, and also on  
20 immunocytochemistry performed after preparation of tissue sections, cytopins or smears. Morphologically it can be difficult to determine the nature of the cells, and as previously mentioned immunocytochemistry can maximally detect the presence of two markers. With the new method, cells from the samples are dispersed e.g. in physiological saline or medium,  
25 incubated with relevant antibody-microsphere combinations for the necessary length of time, usually 30 minutes, and then examined microscopically. In the cell suspension, the bound particles can easily be recognized, permitting a suprisingly rapid and simple immunological fingerprinting or profile of the target cells. Because of this simplicity, the multiparameter characterization,  
30 the very short time frame needed to complete both the procedure and evaluation, the method represents a major contribution in the efforts to achieve rapid and reliable diagnosis of disease and obtain information of crucial importance for the further handling of patients.

35 To illustrate situations where such characterization is important the following teoretical examples are included:



In breast cancer it is known that the expression on the tumor cells of markers such as erbB2, EGF receptor, and IGF2 may be associated with increased proliferation and aggressiveness of the disease. In addition, the expression of other determinants such as EGP2, uPAR, VEGF, MUC1, MDR, Fas, and FasL can reflect characteristics that are important for the ability of the cells to metastasize, to induce angiogenesis, to resist chemotherapy, as well as for apoptosis of the tumor cells or the host T-cells. By using a combination of microspheres several of these parameters can be registered simultaneously in only one operation. Such studies can be performed on the biopsies from the primary tumor, on needle biopsies from solid metastases, and on samples from ascitic or pleural effusions, blood, and bone marrow.

In HIV-infected patients, the characterization of the different subsets of T lymphocytes is of vital importance. Examples of determinants that with the new method can be studied in addition to the most common T-cell markers are chemokine receptors and apoptosis-related molecules such as Fas and FasL.

In malignant melanoma the degree or lack of differentiation of the tumor cells may reflect the potential aggressiveness of the disease in the way that lesser differentiation is related to increased malignancy. In addition, several molecules are important for immunological response, including markers such as gp100, MAGE1, 2, 3, B7, Fas and FasL. Since such markers are important for the effect of immunotherapy and vaccination, comprehensive characterization of these as well as other melanoma-associated antigens are of great importance for the clinical management of the patients. Such characterization can readily be done with the new procedure.

Lymph node enlargement can reflect different types of reactive, infectious, or malignant conditions. Thus, it may be important to determine whether such lymph nodes contain tumor cells or not. If tumor cells are present, determination of the type of malignant cells can decisively influence the choice of therapy. One example is lymph node metastasis that could originate from either a small cell lung cancer (EGP2) or a malignant melanoma (HMW250000). With the appropriate choice of antibody-microsphere

conjugates this distinction can easily be made with the new approach within less than one hour.

## 5 EXAMPLES ON THE USE OF THE NEW PROCEDURE

### 1. Specificity testing of antibody-conjugated fluorescent particles in human breast cancer cells.

10 MCF-7 human breast cancer cells were incubated with 1  $\mu$ m bright pink fluorescent microspheres coated with avidin, with or without biotin-conjugated MOC31 anti-EGP2 (anti-epithelial cell marker) antibody, and/or with immunomagnetic beads (4,5  $\mu$ m) coated with an anti-breast mucin (MUC1) antibody (BM7).

15 A suspension of MCF-7 cells incubated with fluorescent particles without bound MOC31 antibody was examined in a microscope. No fluorescent beads were attached to the tumor cells. Similar experiments with MOC31 biotin-avidin-conjugated fluorescent particles showed from 5 to 10 fluorescent  
20 particles bound to the surface of the tumor cells. In other experiments, MCF-7 cells were incubated with immunomagnetic beads coated with the BM7 antibody that bind to the tumor cells, followed by incubation with fluorescent particles with and without MOC31 antibody. It was found that the tumor cells with surface bound immunomagnetic beads showed binding also and only of  
25 MOC31-conjugated fluorescent particles. The two types of particles could easily be used in parallel, and the results showed no unspecific cell adherence of particles lacking targeting antibody.

### 30 2. Effect of simultaneous or subsequent incubation with antibody-coated beads

Human SKBr3 breast cancer cells were incubated with various combinations of bright pink fluorescent latex microspheres conjugated with MOC31  
35 antibody, with or without simultaneous or subsequent incubation with immunomagnetic beads coated either with MOC31 or with BM7 antibodies.

Bead sizes as in example 1. If both the fluorescent and immunomagnetic beads had the same targeting antibody and were incubated simultaneously, both types of beads were seen bound to the tumor cells. If either of these microspheres/beads were incubated first for 30 min and thereafter for another 30 min with the other antibody-conjugated particles, the binding of the second antibody-particle complex was completely blocked. The binding of each type of beads conjugated with different antibodies and incubated simultaneously showed the same binding pattern as that seen if each of them were studied in separate experiments.

3. Simultaneous binding of several types of microspheres/beads to the same target cells.

Breast cancer cells known to express a number of different antigens on their surface were incubated simultaneously with antibodies recognizing four different of these antigens. Each antibody had independently been conjugated to four types of microspheres/beads: 1) blue dyed latex microspheres (0.5 $\mu$ m), 2) bright pink fluorescent latex microspheres (1  $\mu$ m), 3) yellow fluorescent microspheres (1 $\mu$ m), and 4) immunomagnetic super-paramagnetic particles (4,5  $\mu$ m). The method according to the invention showed that the tumor cells did bind all the four different types of beads which could be clearly recognized by using a combination of fluorescence and light microscopy. The number of particles attached to each cell varied for each antibody-particle complex in accordance with the known expression pattern of the corresponding antigen. The antibodies recognized the following antigens: EGP2, MUC1, EGF receptor, and an independent epithelial marker recognized by the 595 antibody.

4. Binding of fluorescent microspheres on target cells after immunomagnetic enrichment.

MCF7 human tumor cells were added to mononuclear peripheral blood cells from healthy volunteers in a ratio of 1/1000 tumor cells to mononuclear cells. The cell suspension was incubated with MOC31 anti-epithelial antibody attached to bright pink fluorescent microspheres (1  $\mu$ m) through an

avidin/biotin bond and simultaneously with super-paramagnetic immunobeads (4,5  $\mu$ m) coated with the BM7 anti-MUC1 antibody. After 30 min of incubation magnetic selection of tumor cells with immunomagnetic beads bound to their surface was performed, and samples of the resulting cell suspension was examined microscopically. It was found that the remaining tumor cells with bead rosettes on their surface had also bound 5-10 fluorescent particles to the membrane, whereas a contaminating normal cells did not show binding of any of the particles/beads.

5. Binding of fluorescent cells to malignant ascitic cells

A suspension of ascitic cells drawn from a patient was brought to the laboratory without any information of the origin of the cells. The cell suspension was incubated with different antibody-coated fluorescent particles and paramagnetic immunobeads according to the invention. Particles coated with antibodies recognizing different marker antigens bound to the cells in the suspension, demonstrating the malignant and epithelial nature of the cells, thus confirming the diagnosis of ovarian cancer. In another case with ascitic fluid no cells with antibody-coated particles were seen, in agreement with the conclusion of the referring pathologist. In both cases, the method described in the invention provided the results on several different markers within 45 minutes, whereas the parallel morphological and immunocytochemical examination of a single marker was first completed more than 24 hours later.

6. Detection of cells in a pleural effusion

Without any prior knowledge of the underlying disease, incubation of the cell suspension with different anti-tumor antibodies coated onto fluorescent and immunomagnetic particles showed strong binding of all microspheres and immunobeads with anti-carcinoma and breast mucin (MUC1) antibodies. The diagnosis of the patient was breast cancer with pleural effusion. Microspheres conjugated with an anti-melanoma antibody did not bind. In conclusion, also in this case the cells in the clinical sample were successfully identified.

7. Needle aspirate from a thyroid tumor

- 5 The cell suspension obtained from needle aspirate was incubated with  
fluorescent microspheres with an antibody known to react with colorectal  
cancer cells, and simultaneously with immunomagnetic beads coated with the  
MOC31 anti-epithelial antibody. The fluorescent microspheres did not bind  
10 to any types of cells in the suspension, whereas the MOC31 immunobeads  
bound strongly to thyroid epithelial cells but not to the high number of  
macrophages present in the suspension.

The above examples demonstrate that the new method according to the  
invention shows considerably increased diagnostic strength and reliability in  
that a higher number of target cell antigenic determinants can be deleted in  
15 one operation, in a very short period of time compared to previously known  
methods.

## CLAIMS

1. Method to detect and phenotype target cells in cell suspensions by using particles coated with antibodies/ligands directed against antigenic determinants/receptors expressed on the target cells,  
5 **characterized in** that several types of particles, each particle instrumentally or visually separable by fluorescence, color and size, and each type of particle coated with a different antibody or ligand, are incubated simultaneously or subsequently with cell suspensions containing the target cells, in connection or not with a per se known enrichment procedure.
- 10 2. Method according to claim 1,  
**characterized in** that the particles used in the method are separable by a combination of fluorescence, color and/or size or a combination of fluorescent emission spectra, different colors or different sizes, preferably fluorescent emission spectra and/or size.
- 15 3. Method according to claim 1-2,  
**characterized in** that the size of the particles used in the method ranges from 0,01  $\mu\text{m}$  to 6  $\mu\text{m}$ , preferably from 0,5  $\mu\text{m}$  to 4,5  $\mu\text{m}$ .
4. Method according to claim 1-3,  
**characterized in** that it comprises the following steps;  
20 - adding several antibodies, ligands preferably 2-6, each antibody conjugated to specific separably particles, to the cell suspension containing the target cells, ratio 20:1 to 0.5:1, preferably 5:1 (number of particles/number of cells), limited by the size of the particles,  
- incubate under gentle rotation for 5-10 minutes to 2 hours, preferably 30  
25 minutes, at 0°C to 37°C, preferably 4°C,  
- evaluation of the target cell rosettes microscopially and/or by suitable visualizing or imaging devices.
5. Method according to claim 1-4,  
**characterized in** that the particles used in the method are coated with  
30 ligands/antibodies directed against adhesion molecules, carbohydrate antigens, glycolipids, growth factor receptors, melanoma antigens, sarcoma antigens, carcinoma markers, neuroblastoma antigens, glioma antigens, head and neck antigens, HLA-antigens, apoptosis-associated molecules, motility-

related antigens, proliferation-associated antigens, differentiation-associated antigens, drug resistant-related antigens, angiogenesis-associated antigens, chemokine receptors, invasion-related antigens, B- and T-cell CD antigens and various antigens, such as cathepsin D, neuroglandular antigen (CD63) and pan-human antigen.

6. Method according to claim 5, characterized in that the particles used in the method are coated with ligands/antibodies directed against the receptors/antigens listed in Table 1.

7. Method according to claims 5-6, characterized in that the particles used in the method are coated with antibodies such as MOC31 anti EGP2 (anti-epithelial cell marker) antibody, anti-breast mucin (MUC1) antibody (BM7), 595, anti-EGF receptor (425.3), anti-erbB2 and anti-HMW melanoma antigen (9.2.27).

8. Use of the method according to claim 1-7, wherein it is performed phenotyping of the target cells comprising profiling the antigenic determinants or receptors expressed on the cell membrane of the target cells.

9. Use according to claim 8, wherein the target cell characteristics of biologically informative markers of diagnostic, prognostic and therapeutic value are registered.

10. Use according to claim 9, wherein the target cells are malignant cells and/or HIV-infected T-lymphocytes.

11. Use according to claim 9, wherein the biologically informative markers are adhesion molecules, such as E-cadherin, CD44 variants, NCAM (CD56), growth factor receptors such as EGF, carcinoma marker such as EGP2, carbohydrate antigens such as Le<sup>y</sup>, CEA, 15-3 epitope, growth factor receptor such as EGFr, c-erbB2, IL-2 receptor, TNF receptor, melanoma antigens such as HMW 250000, gp 75/TRP-1, p95, MAG 1, 2, 3, sarcoma antigens such as TP 1 and TP 3 epitopes, carcinoma markers such as EGP2, MUC1, MUC2 & 3, PSA, PSM, GA733.2, TAG72, 15-3 epitope, ovarian carcinoma DC 125 epitope, glioma antigens such as Mel-14 epitope, HLA antigens such as HLA class I, HLA-DR, DQ, DP,  $\beta_2$ -microglobulin, apoptosis associated markers such as Fas, FasL, p75, motility related markers such as KAT-1, AMF, proliferation associated antigens such as gp120,

differentiation associated markers such as MUC 18, TA99, drug resistance markers such as MDR, MRP, angiogenesis associated antigens such as VEGF, bFGF, chemokine receptors such as CCR, CXCR, invasion-related markers such as uPAR, uPA, PAT-1, TIMP1 & 2 MMP9, stromelysins, B- and T-cell antigens such as CD2, CD10, CD4, CD8, CD19, CD20, CD22, CD37, and other antigens such as cathepsin D and par-human epitope.

12. Kit to perform the method according to claim 1-7, characterized in in that it comprises particles conjugated to antibodies/ligands according to claims 5-7.



# INTERNATIONAL SEARCH REPORT

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International application No.  
PCT/NO 97/00342

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/543, G01N 33/569  
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	WO 9407142 A1 (SRI INTERNATIONAL), 31 March 1994 (31.03.94), see page 4-7, 12, 21-22, 36-38, 40 --	1-12
X	US 5326696 A (TSE W. CHANG), 5 July 1994 (05.07.94), see column 9-10 and claims --	1-3,5-12
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☒ Further documents are listed in the continuation of Box C.

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| <ul style="list-style-type: none"> <li>* Special categories of cited documents:</li> <li>"A" document defining the general state of the art which is not considered to be of particular relevance</li> <li>"E" earlier document but published on or after the international filing date</li> <li>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</li> <li>"O" document referring to an oral disclosure, use, exhibition or other means</li> <li>"P" document published prior to the international filing date but later than the priority date claimed</li> </ul> | <ul style="list-style-type: none"> <li>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</li> <li>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</li> <li>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</li> <li>"&amp;" document member of the same patent family</li> </ul> |
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International application No.

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X	US 5194300 A (SAU W. CHEUNG), 16 March 1993 (16.03.93), see examples 4-8 --	1-12
X	US 5374531 A (BRUCE D. JENSEN), 20 December 1994 (20.12.94), see column 18 and claims --	1-12
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International application No.  
PCT/NO 97/00342

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# INTERNATIONAL SEARCH REPORT

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2  
INTERNATIONAL SEARCH REPORT

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

International application No.  
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Information on patent family members

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